VARIANTS OF SOLUBLE PYRROLOQUINOLINE QUINONE-DEPENDENT GLUCOSE DEHYDROGENASE

The present invention relates to improved variants of soluble pyrroloquinoline quinone (PQQ)-dependent glucose dehydrogenases (s-GDH), to genes encoding mutated s-GDH, to mutant proteins of s-GDH with improved substrate specificity for glucose, and to different applications of these s-GDH variants, particularly for determining concentrations of sugar, especially of glucose in a sample.

Two types of PQQ-dependent glucose dehydrogenase (EC 1.1.99.17) have been characterized: One is membrane-bound (m-GDH), the other is soluble (s-GDH). Both types do not share any significant sequence homology (Cleton-Jansen, A. M., et al., Mol Gen Genet 217 (1989) 430-6; Cleton-Jansen, A. M., et al., Antonie Van Leeuwenhoek 56 (1989) 73-9; Oubrie, A., et al., Proc Natl Acad Sci U S A 96 (1999) 11787-91). They are also different regarding both their kinetic as well as their immunological properties (Matsushita, K., et al., Bioscience Biotechnology & Biochemistry 59 (1995) 1548-1555).

Quinoproteins use quinone as cofactor to oxidize alcohols, amines and aldoses to their corresponding lactones, aldehydes and aldolic acids (Duine, J. A. Energy generation and the glucose dehydrogenase pathway in *Acinetobacter* in "The Biology of *Acinetobacter*" (1991) 295-312, New York, Plenum Press; Duine, J. A., Eur J Biochem 200 (1991) 271-84, Davidson, V. L. - in "Principles and applications of quinoproteins" (1993) the whole book, New York, Marcel Dekker; Anthony, C., Biochem J 320 (1996) 697-711 ;Anthony, C. and Ghosh, M., Current Science 72 (1997) 716-727; Anthony, C., Biochem Soc Trans 26 (1998) 413-7; Anthony, C. and Ghosh, M., Prog Biophys Mol Biol 69 (1998) 1-21). Among quinoproteins, those containing the noncovalently bound cofactor 2,7,9-tricarboxy-1H-pyrrolo [2,3-f]quinoline-4,5-dione (PQQ) constitute the largest sub-group (Duine 1991, supra). All bacterial glucose dehydrogenases known so far belong to this sub-group with PQQ as the prosthetic group (Anthony and Ghosh 1997, supra, Goodwin and Anthony 1998, supra).

In bacteria, there are two completely different types of PQQ-dependent glucose dehydrogenases (EC1.1.99.17): the soluble type (s-GDH) and the membrane-bound type

(m-GDH) (Duine et al., 1982; Matsushita et al., 1989a,b). The m-GDHs are widespread in Gram-negative bacteria, s-GDHs, however, have been found only in the periplasmatic space of *Acinetobacter* strains, like *A. calcoaceticus* (Duine, 1991a; Cleton-Jansen et al., 1988; Matsushita and Adachi, 1993) and *A. baumannii* (JP 11243949).

Through searching sequence databases, two sequences homologous to the full-length *A. calcoaceticus* s-GDH have been identified in *E.coli* K-12 and *Synechocystis sp.*. Additionally, two incomplete sequences homologous to *A. calcoaceticus* s-GDH were also found in the genome of *P.aeruginosa* and *Bordetella pertussis* (Oubrie et al. 1999a), respectively. The deduced amino acid sequences of these four uncharacterized proteins are closely related to *A. calcoaceticus* s-GDH with many residues in the putative active site absolutely conserved. These homologous proteins are likely to have a similar structure and to catalyze similar PQQ-dependent reactions (Oubrie et al., 1999a).

Bacterial s-GDHs and m-GDHs have been found to possess quite different sequences and different substrate specificity. For example, *A. calcoaceticus* contains two different PQQ-dependent glucose dehydrogenases, one m-GDH which is active *in vivo*, and the other designated s-GDH for which only *in vitro* activity can be shown. Cleton-Jansen et al., (1988;1989a,b) cloned the genes coding for the two GDH enzymes and determined the DNA sequences of both these GDH genes. There is no obvious homology between m-GDH and s-GDH corroborating the fact that m-GDH and s-GDH represent two completely different molecules.

The gene of s-GDH from *A. calcoaceticus* has been cloned in *E. coli* behind a leader sequence and a strong promoter. After being synthesized in the cell, the s-GDH is translocated through the cytoplasmic membrane into the periplasmic space (Duine, J. A. Energy generation and the glucose dehydrogenase pathway in *Acinetobacter* in "The Biology of *Acinetobacter*" (1991) 295-312, New York, Plenum Press, Matsushita, K. and Adachi, O. Bacterial quinoproteins glucose dehydrogenase and alcohol dehydrogenase in "Principles and applications of Quinoproteins" (1993) 47-63, New York, Marcel Dekker). Like the native s-GDH from *A. calcoaceticus*, s-GDH expressed in *E.coli* is also a homodimer, with one PQQ molecule and three calcium ions per monomer (Dokter et al., 1986 supra,1987 supra1988 supra; Olsthoorn, A. and J. Duine, J. A., Arch Biochem Biophys 336 (1996) 42-8; Oubrie, A., et al., J Mol Biol 289 (1999) 319-33, Oubrie, A., et al., Proc Natl Acad Sci U S A 96 (1999) 11787-91, Oubrie, A., et al., Embo J 18 (1999) 5187-94). s-GDH oxidizes a wide range of mono- and disaccharides to the corresponding ketones which further hydrolyze to

the aldonic acids, and it is also able to donate electrons to PMS (phenazine metosulfate), DCPIP (2,6-dichlorophenolindophenol), WB (Wurster's blue) and short-chain ubiquinones such as ubiquinone Q1 and ubiquinone Q2 (Matsushita, K., et al., Biochemistry 28 (1989) 6276-80; Matsushita, K., et al., Antonie Van Leeuwenhoek 56 (1989) 63-72), several artificial electron acceptors such as N-methylphenazonium methyl sulfate (Olsthoorn, A. J. and Duine, J. A., Arch Biochem Biophys 336 (1996) 42-8 ; Olsthoorn, A. J. and Duine, J. A., Biochemistry 37 (1998) 13854-61) and electroconducting polymers (Ye, L., et al., Anal. Chem. 65 (1993) 238-41).

In view of s-GDH's high specific activity towards glucose (Olsthoorn, A. J. and Duine, J. A., Arch Biochem Biophys 336 (1996) 42-8) and its broad artificial electron acceptor specificity, the enzyme is well suited for analytical applications, particularly for being used in (bio-)sensor or test strips for glucose determination in diagnostic applications (Kaufmann et al., 1997 supra).

Glucose oxidation can be catalyzed by at least three quite distinct groups of enzymes, i.e., by NAD-dependent, dye-linked glucose dehydrogenases, by flavoprotein glucose oxidase or by quinoprotein GDHs (Duine 1995). A rather slow autooxidation of reduced s-GDH has been observed, demonstrating that oxygen is a very poor electron acceptor for s-GDH (Olsthoorn and Duine 1996). s-GDH can efficiently donate electrons to PMS, DCPIP, WB and short-chain ubiquinones such as Q1 and Q2, but it can not efficiently donate electrons directly to oxygen.

Traditional test strips and sensors for monitoring glucose level in blood, serum and urine e. g. from diabetic patients use glucose oxidase. However, since glucose oxidase transfers its electrons to oxygen, it is known that oxygen may have a negative impact on glucose measurements which are based on this enzyme. The major advantage of PQQ-dependent glucose dehydrogenases is their independence from oxygen. This important feature is e.g., discussed in US 6,103,509, in which some features of membrane-bound GDH have been investigated.

An important contribution to the field has been the use of s-GDH together with appropriate substrates. Assay methods and test strip devices based on s-GDH are disclosed in detail in US 5,484,708. This patent also contains detailed information on the set-up of assays and the production of s-GDH-based test strips for measurement of glucose. The methods described there as well in the cited documents are herewith included by reference.

Other patents or applications relating to the field and comprising specific information on various modes of applications for enzymes with glucose dehydrogenase activity are US 5,997,817; US 6,057,120; EP 620 283; and JP 11-243949-A.

A commercial system which utilizes s-GDH and an indicator that produces a color change when the reaction occurs (Kaufmann et al. 1997) is the Glucotrend® system distributed by Roche Diagnostics GmbH.

Despite the above discussed important advantages there also is a major inherent problem of s-GDH. s-GDH has rather a broad substrate spectrum as compared to m-GDH. That is, s-GDH oxidizes not only glucose but also several other sugars including maltose, galactose, lactose, mannose, xylose and ribose (Dokter et al. 1986a). The reactivity towards sugars other than glucose may in certain cases impair the accuracy of determining blood glucose levels, in some diabetic patients. In particular patients on peritoneal dialysis treated with icodextrin (a glucose polymer) may contain in their body fluids, e.g., in blood, high levels of other sugars, especially maltose (Wens, R., et al., Perit Dial Int 18 (1998) 603-9).

Therefore clinical samples, as e.g. obtained from diabetic patients, especially from patients with renal complications and especially from patients under dialysis may contain significant levels of other sugars, especially maltose. Glucose determinations in samples obtained from such critical patients may be impaired by maltose.

There are scarce reports in the literature on attempts to produce modified PQQ-dependent s-GDHs which exhibit altered substrate specificity. Due to a negative outcome most of these efforts have not been published. Igarashi, S., et al., (1999) report that introducing a point mutation at position Glu277 leads to mutants with altered substrate specificity profile. However, none of these mutants, lead to an at least two-fold increased improved specificity for glucose as e.g., compared to xylose, galactose or maltose.

It can be summarized that the attempts known in the art aiming at improvements of properties of s-GDH, especially its specificity towards glucose, have not been successful to the extend required for accurate monitoring of glucose levels in patients having also high levels of sugars other than glucose.

A great demand and clinical need therefore exists for mutant forms of s-GDH which feature an improved specificity for glucose as substrate.

It was the task of the present invention to provide new mutants or variants of s-GDH with significantly improved substrate specificity for glucose as compared to other selected sugar molecules, e.g., like galactose or maltose.

Surprisingly it has been found that it is possible to significantly improve the substrate specificity of s-GDH for glucose, as compared to other sugars, and to at least partially overcome the above described problems known in the art.

The substrate specificity for glucose as compared to other selected sugar molecules has been significantly improved by providing mutant s-GHD according to the present invention as described herein below and in the appending claims. Due to the improved substrate specificity of the new forms of s-GDH, significant technical progress for glucose determinations in various fields of applications is possible.

Summary of the invention:

It has now surprisingly been found that it is possible to provide s-GDH mutants with improved substrate specificity towards glucose as a substrate as compared to other selected sugars. New s-GDH variants are disclosed which exhibit a significant higher substrate specificity for glucose, especially as compared to maltose.

Also disclosed are mutant s-GDH molecules, which, compared to the wild-type enzyme, exhibit essentially equal specific activity for glucose as substrate but markedly reduced activity for other selected sugar molecules.

Such comparison of a mutant's specific activity for one of the various other substrate molecules is based on and calculated in relation to the original enzymatic activities of a wild-type enzyme, e.g., as isolated from *Acinetobacter calcoaceticus*.

Mutated s-GDH proteins as well as polynucleotide-sequences coding for such proteins exhibiting improved properties, especially increased specificity for glucose are also provided.

s-GDH mutants comprising at least one amino acid substitutions at an amino acid position corresponding to a position of the s-GDH wild-type sequence known from *A. calcoaceticus* (SEQ ID NO: 24) selected from the group consisting of positions 348 and 428 have been

found to provide for s-GDH enzymes with improved properties, especially with improved specificity for glucose. Variants comprising a substitution at position 348 exhibit a strikingly positive effect on glucose specificity.

The improved s-GDH mutants can be used with great advantage for the specific detection or measurement of glucose in biological samples, especially in tests strip devices or in biosensors.

The following examples, references, sequence listing and figures are provided to aid the understanding of the present invention, the true scope of which is set forth in the appended claims. It is understood that modifications can be made in the procedures set forth without departing from the spirit of the invention.

Description of the Figures

Figure 1: Nucleotide (DNA) sequence of the *Acinetobacter calcoaceticus* PQQ dependent soluble glucose dehydrogenase gene and the corresponding amino acid sequence (SEQ ID NO: 23).

Figure 2: Protein sequences of *A. calcoaceticus* PQQ-dependent s-GDH (top) and *A. baumannii* s-GDH (bottom) aligned according to sequence homology.

Figure 3: Illustration of pACSGDH vector referred to in Example 1 containing the wild-type or mutated DNA sequences of soluble PQQ-dependent glucose dehydrogenase.

Figure 4: Nucleotide (DNA) sequence of the pACSGDH vector referred to in Example 1 containing the wild-type DNA sequence of soluble PQQ-dependent glucose dehydrogenase (SEQ ID NO: 25).

Detailed description of the invention:

In a first embodiment the invention relates to a mutant of the soluble form of EC 1.1.99.17 also known as PQQ-dependent soluble glucose dehydrogenase (s-GDH), said mutant characterized in that relative to the corresponding wild-type enzyme and with regard to at least one other selected sugar substrate, it has an at least two-fold improved substrate specificity for glucose .

The directions given in this invention are easily applied for any known or even yet unknown isolate of s-GDH. These wild-type isolates can be used to assess the relative improvements in specificity for the variants generated therefrom.

The wild-type enzyme of *Acinetobacter calcoaceticus*-type strain LMD 79.41 which corresponds to SEQ ID NO: 24 is well-known and well-characterized. In case a different wild-type enzyme under investigation is not well-characterized it is advantageous to use the s-GDH from *Acinetobacter calcoaceticus*-type strain LMD 79.41 as a reference. In a further preferred embodiment the properties of an improved variant of s-GDH are compared to this wild-type enzyme. The present invention therefore also relates to a mutant of s-GDH said mutant characterized in that relative to the wild-type enzyme of SEQ ID NO: 24 and with regard to at least one other selected sugar substrate, it has an at least two-fold increased substrate specificity for glucose.

As discussed above, two completely different enzyme families with glucose dehydrogenase activity, grouped together under EC1.1.99.17, are characterized to date. These two enzyme families, however, appear not be related to each other.

For the purpose of this invention only the soluble form of GDH (s-GDH) is relevant and improved variants thereof are discussed herein below.

It is known in the art that the wild-type DNA-sequence of a soluble PQQ-dependent glucose dehydrogenase can be isolated from strains of *Acinetobacter*. Most preferred is the isolation from *Acinetobacter calcoaceticus*-type strain LMD 79.41. The sequence of this wild-type s-GDH (the mature protein) is given in Figure: 1 and SEQ ID NO: 24. Other LMD strains of *Acinetobacter* may also be used as source of wild-type s-GDH. Such sequences can be aligned to the sequence obtained from *A. calcoaceticus* and sequence comparisons be made (see Figure: 2). It also appears feasible to screen DNA-libraries of other bacterial strains, as for example described for *E.coli* K-12 (Oubrie, A., et al., J Mol Biol 289 (1999) 319-33) above and to identify sequences related to s-GDH in such genomes. Such sequences and yet unidentified homologous sequences may be used to generate s-GDH mutants with improved substrate specificity for glucose.

The term "mutant" or "variant" in the sense of the present invention relates to a s-GDH protein which compared to a corresponding wild-type sequence exhibits at least one amino acid substitution. The expert in the field will appreciate that there are various possibilities

to produce polynucleotides encoding for a polypeptide sequence of such mutated s-GDH. It is of course also possible to generate mutants comprising one or more additions or deletions of amino acids.

A mutant according to the present invention is characterized in that relative to the corresponding wild-type enzyme it has at least a two-fold improved substrate specificity for glucose as compared to at least one other selected sugar substrate.

In order to calculate the substrate specificity or cross-reactivity one easy way is to set the activity measured with glucose as substrate to 100% and to compare the activity measured with the other selected sugar to the glucose value. Sometimes, in order not to be redundant, simply the term specificity is used without making special reference to glucose on the one hand and a selected other sugar substrate on the other hand.

The expert in the field will appreciate that comparison of (re-)activities is best made at equimolar concentrations of the substrate molecules investigated using well-defined assay conditions. Otherwise corrections for differences in concentrations have to be made.

Standardized and well-defined assay conditions have to be chosen in order to assess (improvements in) specificity. The enzymatic activity of s-GDH for glucose as substrate as well as for other selected sugar substrates is measured as described in Example 6.

Based on these measurements cross-reactivity and (improvements in) specificity are assessed.

The s-GDH (cross-)reactivity for a selected sugar in percent is calculated as

Cross-reactivity [%] = (activity selected sugar/activity glucose) x 100%.

(Cross-)reactivity for maltose of wild-type s-GDH according to the above formula has been determined as about 105%. Wild-type s-GDH (cross-)reactivity for galactose has been measured as about 50% (cf. Table 1).

(Improved) specificity is calculated according to the following formula:

$$\text{specificity (improvement)} = \frac{\text{activity glucose mutant}}{\text{activity glucose wild-type}} \times \frac{\text{activity selected sugar wild-type}}{\text{activity selected sugar mutant}}$$

As compared to the wild-type enzyme, a s-GDH form with an at least two-fold improvement in specificity for glucose versus maltose (maltose/glucose) accordingly with maltose as substrate has at most 52,5% of the activity as measured with glucose as substrate. Or, if, for example, a mutant s-GDH has a cross-reactivity for maltose of 20 % (determined and calculated as described above), this mutant as compared to the wild-type s-GDH therefore has a 5.25 fold improved substrate specificity (maltose/glucose).

The term "specific activity" for a substrate is well known in the art, it is preferably used to describe the enzymatic activity per amount of protein. Various methods are known to the art to determine specific activity of GDH molecules, using glucose or other sugars as substrates (Igarashi, S., et al., Biochem Biophys Res Commun 264 (1999) 820). One of the methods available for such measurement is described in detail in the examples section.

Whereas it is possible, to select many different sugar molecules and to investigate the glucose specificity of s-GDH in comparison to any such selected sugar molecule, it is preferred to select a clinically relevant sugar molecule for such a comparison. Preferred selected sugars are selected from the group consisting of mannose, allose, galactose, xylose, and maltose. More preferably, maltose or galactose are selected and mutant s-GDH is tested for improved substrate specificity for glucose as compared to galactose or maltose. In a further preferred embodiment the selected sugar is maltose.

It has surprisingly been found that the improvements in glucose specificity of mutated s-GDH, e.g., for maltose vs. glucose, are quite considerable. It is therefore further preferred that said substrate specificity for glucose as compared to the substrate specificity for at least one of the selected other sugar substrates is improved at least three-fold. Other preferred embodiments comprise s-GDH mutants characterized by an improved substrate specificity for glucose, which is at least 5 times higher or also preferred at least 10 times higher, as compared to the other sugar molecule selected.

Mutations in s-GDH lead in many cases to enzyme variants with drastically reduced specific activity for the substrate glucose. Such decrease in (absolute or overall) specific activity for the substrate glucose, however, may be critical for routine applications. Surprisingly it has been found that improved specificity for glucose must not go to the expense of a dramatically reduced overall specific activity. It is therefore preferred that the s-GDH with improved specificity towards the substrate glucose exhibits at least 10% of the specific activity for glucose as measured with the wild-type enzyme. It is of course more preferred that such mutated enzymes exhibit at least 20% or more preferred at least 50% of the respective glucose activity of wild-type s-GDH.

In a further preferred embodiment, the invention relates to a mutant of the soluble form of EC1.1.99.17 also known as PQQ-dependent soluble glucose dehydrogenase (s-GDH), said mutant characterized in that

a) the substrate specific reactivity towards glucose is essentially equal to that of the wild-type enzyme and

b) the substrate specific reactivity towards maltose is 30% or less as compared to the wild-type enzyme.

The substrate specific reactivity (also termed specific activity) towards glucose is considered to be essentially equal to that of the wild-type enzyme if at least 50% of the original enzymatic activity for glucose of the wild-type enzyme is maintained. Also preferred are mutants exhibiting at least 80 % or more preferred, at least 90 % of the specific activity for glucose as measured for the wild-type enzyme.

Quite surprisingly it has been found, that it is possible to obtain such s-GDH mutants or variants, which compared to the wild-type s-GDH exhibit essentially equal enzymatic activity for glucose but, nonetheless, significantly reduced substrate specific reactivity towards other selected sugars, especially towards maltose. Mutants characterized in that the substrate specific reactivity towards glucose is essentially equal to that of the wild-type enzyme and in that the substrate specific reactivity towards maltose is 20% or less as compared to the wild-type enzyme are preferred. Further preferred are such mutants for which the maltose specific activity is 15 % or even only 10% or less of the maltose specific activity as measured for the corresponding wild-type enzyme, whereas the specific

reactivity for glucose is essentially equal to the specific activity for glucose of the corresponding wild-type enzyme.

In case the wild-type enzyme is not well-characterized it is preferred to use the s-GDH from *Acinetobacter calcoaceticus*-type strain LMD 79.41 as reference. In a further preferred embodiment the properties of an improved variant of s-GDH are compared to this wild-type enzyme and the present invention therefore relates to a mutant of s-GDH said mutant characterized in that relative to the wild-type enzyme of SEQ ID NO: 24 it exhibits essentially equal enzymatic activity for glucose but, significantly reduced (at least 70% less) substrate specific reactivity towards at least one other selected sugar substrate.

Unexpectedly it has been found that it is possible to generate s-GDH mutants with improved substitute specificity and even more unexpectedly it has been found that it is only a few well-defined amino acid positions which are of major relevance in that respect.

The achievements of the present invention are described in great detail by making reference to amino acid positions known from SEQ ID NO: 24, the wild-type sequence of s-GDH as isolated from *Acinetobacter calcoaceticus*-type strain LMD 79.41. Amino acid positions in different s-GDH isolates corresponding to positions of SEQ ID NO: 24 are easily identified by appropriate sequence comparison. Preferably the PileUp program is used to assess homology or identity between such sequences. The amino acid positions given herein below shall be understood as amino acid positions of SEQ ID NO: 24 or of positions corresponding thereto in other s-GDH molecules, unless specific reference is made to a different SEQ ID NO or to a different s-GDH isolate. In order to avoid redundances only in a few cases a specific hind to the fact that corresponding positions in different isolates can be modified in the same way is given, whereas for convenience in most cases simply and only the corresponding position from SEQ ID NO: 24 is used.

Mutants comprising an amino acid substitution at the position corresponding to position 348 of s-GDH have been found to exhibit a striking effect on specificity for glucose. As demonstrated in table 1, a variety of s-GDH variants with improved specificity for glucose can be identified and generated, as long as at least the amino acid in position threonine 348 – as corresponding to wild-type s-GDH sequence position from *A. calcoaceticus* – is substituted with an appropriate other amino acid.

A preferred embodiment of the present invention therefore relates to a mutant protein of PQQ-dependent s-GDH comprising an amino acid residue substitution at the amino acid position corresponding to position 348 of the s-GDH wild-type sequence known from *A. calcoaceticus* (SEQ ID NO: 24).

It has also been found, that combined substitutions of amino acids at amino acid positions corresponding to positions 348 and 428 of SEQ ID NO: 24 are advantageous to generate s-GDH mutants or variants with significantly improved specificity for glucose.

Residues 348 or 428 of s-GDH as isolated from *Acinetobacter calcoaceticus*-type strain LMD 79.41 are not known from the art to contribute to the substrate binding of s-GDH (Oubrie, A., et al., Embo J 18 (1999) 5187-94, ; Oubrie, A. and Dijkstra, B. W., Protein Sci 9 (2000) 1265-73). No chemical or physical explanation is at hand, why substitutions of these amino acid residues alters the substrate specificity of s-GDH for glucose as compared to other sugar molecules of interest.

It has been found in addition that substitution of amino acid 76 also has a positive effect on glucose specificity of s-GDH.

In a further preferred embodiment the mutated s-GDH is characterized in that the amino acid residue threonine at position 348 is substituted with an amino acid residue selected from the group consisting of alanine, glycine, and serine. In a most preferred embodiment glycine is used to substitute for threonine at position 348.

One group of preferred s-GDH variants according to this invention comprises a substitution of the amino acid residue at position 348 and at at least one of the following positions 76, 143, 168, 169 and 428.

In yet a further embodiment a mutant protein of PQQ-dependent s-GDH comprises an amino acid residue substitution at position 428 of the correspondent wild-type sequence from *Acinetobacter calcoaceticus*, in which the asparagine residue of the wild-type sequence is replaced by other appropriate amino acid residues. Preferably, such amino acid residue is selected from the group consisting of leucine, proline and valine. It is preferred to substitute the asparagine at position 428 with proline .

It further has proved that the amino acid glutamine at position 76 can be substituted to improve on the problems imposed by s-GDH cross-reactivity with other sugar molecules. Sequence positions of other s-GDH isolates corresponding to this sequence position are easily identified by a homology search based on SEQ ID NO:3. In another preferred embodiment the mutant according to the present invention therefore comprises a substitution of glutamine at position 76 of the corresponding wild-type sequence from *Acinetobacter calcoaceticus*.

It is preferred to select the amino acid used in such substitution at a position corresponding to position 76 in SEQ ID NO: 24 from the group consisting of alanine, aspartic acid, glutamic acid, glycine, methionine, proline and serine.

As described above, a substitution of the amino acid in position 348 of the s-GDH sequence corresponding to the wild-type sequence isolated from *A. calcoaceticus*, can be used to significantly improve the glucose specificity of s-GDH. Further improved mutants are obtained by providing a mutant s-GDH protein comprising at least two amino acid substitutions, wherein the amino acid corresponding to amino acid position 348 of SEQ ID NO : 24 is substituted.

A further embodiment of the present invention therefore is a mutant s-GDH comprising at least two amino acid residue substitutions at an amino acid position corresponding to a position of the s-GDH wild-type sequence known from *A. calcoaceticus* (SEQ ID NO: 24), said substituted amino acid positions being selected from the group consisting of positions 16, 22, 76, 116, 120 ,127, 143, 168, 169, 171, 177, 227, 230, 231, 245, 255, 277, 295, 299, 308, 317, 341, 348, 349, 355, 422, 428 and 438, wherein the amino acid residue T348 is replaced. It is further preferred, that a s-GDH variant with the at least two substituted amino acid residues comprises a substitution in position 348 and at least one additional substitution selected from the group of positions comprising positions 76, 143, 168, 169, and 428.

In a further preferred embodiment the at least two amino acid positions which are substituted in a mutant s-GDH are selected from the group consisting of amino acid positions 76, 348, and/or 428.

Mutants comprising substitutions at amino acid residues corresponding to positions 348 and 428 have been found as very advantageous for improving the specificity of s-GDH for

glucose in comparison to other sugar substrates. It is especially preferred to design and select mutants of s-GDH, which comprise a substitution at both the positions 348 and 428. Most preferred are mutants comprising the preferred substitutions as described above at both these positions. A s-GDH variant comprising T348G and N428P is most preferred.

The terminology T348G and N428P is known to the art to indicate that threonine at position 348 is replaced by glycine and glutamine and position 428 is replaced by proline.

Mutant s-GDH proteins comprising in addition to substitutions at positions 348 and 428 also substitutions at positions 76, 127 and 143, also represent preferred embodiments of the present invention.

Further preferred examples of mutated s-GDH proteins according to the present invention comprise amino acid substitutions at positions 76 and 348 and also such mutants comprising substitutions at positions 76 and 428. In yet another preferred embodiment the mutated s-GDH protein according to the present invention comprises substitutions of the amino acid residues at positions 76, 348 and 428.

In yet a further preferred embodiment the s-GDH variant according to the present invention comprises at least three amino acid substitutions, the at least three substituted amino acid residues corresponding to amino acid positions of the s-GDH wild-type sequence known from *A. calcoaceticus* (SEQ ID NO: 24), said substituted amino acid positions being selected from the group consisting of positions 171, 227, 230, 245, 341, 348, 349, and 428, wherein both the amino acid residues T348 and N428 are substituted. Preferably such trible mutants comprise at least one of the following substitutions: Y171G, H227F, P230H, E245D or M341V.

Preferred s-GDH variants with highly improved specificity for glucose comprise substitutions at positions 348 and 428 in combination with substitutions at positions 245 or 341, or both.

Amino acid sequence analysis revealed that the sequence motives found in wild-type s-GDH from *A. calcoaceticus* on the one hand and *A. baumannii* on the other hand appear to be very conservative around the positions of major relevance to improve the specificity for

glucose as identified in the present invention, i.e. positions 76, 348, and 428, as corresponding to wild-type s-GDH from *A. calcoaceticus* (c.f., Figure 2).

A preferred embodiment according to the present invention therefore is a mutant protein of PQQ-dependent s-GDH comprising the amino acid sequence of WPXaaVAPS (SEQ ID NO: 1), wherein said Xaa residue is an amino acid residue other than threonine. SEQ ID NO:1 corresponds to position 346-352 of *A. calcoaceticus* wild-type s-GDH or position 347-353 of *A. baumannii* wild-type s-GDH.

Further preferred is an s-GDH mutant wherein said Xaa in SEQ ID NO: 1 represents alanine, glycine or serine, most preferred, Xaa represents glycine.

A mutant of PQQ-dependent s-GDH, comprising the amino acid sequence of TAGXaaVQK (SEQ ID NO: 2), wherein said Xaa residue is an amino acid residue other than asparagine, is another preferred embodiment of the invention. SEQ ID NO:2 corresponds to position 425-431 of *A. calcoaceticus* wild-type s-GDH or to position 426-432 of *A. baumannii* wild-type s-GDH.

Preferably the s-GDH mutant comprising SEQ ID NO:2 is characterized in that said Xaa residue is selected from the group consisting of leucine, proline and valine, most preferred Xaa is a proline residue.

Numerous possibilities are known in the art to produce mutant proteins. Based on the important findings of the present invention disclosing the critical importance of amino acid positions 348 and 428 and also the utility of position 76, the skilled artisan now can easily produce further appropriate variants of s-GDH. Such variants for example can be obtained by the methods known as random mutagenesis (Leung, D. W., et al., Technique 1 (1989) 11-15) and/or directed mutagenesis (Hill, D. E., et al., Methods Enzymol 155 (1987) 558-68). An alternative method to produce a protein with the desired properties is to provide chimaeric constructs, which contain sequence elements from at least two different sources or to completely synthesize an appropriate s-GDH gene. Such procedures known in the art may be used in combination with the information disclosed in the present invention to provide mutants or variants of s-GDH comprising at least one amino acid substitution at a sequence position corresponding to positions 348, and/or 428 of SEQ ID NO: 24.

A s-GDH variant according to the present invention can e.g., be produced by starting from a s-GDH gene as isolated from *Acinetobacter calcoaceticus*-type strain LMD 79.41 as well as by starting from a homologous sequence. In the context of this application the term "homologous" is meant to comprise wild-type s-GDH as isolated from other microorganisms, provided that the sequence homology as compared to SEQ ID NO: 24 is at least 90 %. With other words, after appropriate alignment using the PileUp program, at least 90 % of the amino acids of that s-GDH are identical to the amino acids described in SEQ ID NO: 24.

It will be understood that variations of DNA and amino acid sequences naturally exist, or may be intentionally introduced using methods known in the art. These variations may result in up to 10 % amino acid differences in the overall sequence, due to deletions, substitutions, insertions, inversions or additions of one or more amino acid residues in said sequence as compared to SEQ ID NO: 24. Such amino acid substitutions may be made, for example, on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity and/or the amphipathic nature of the residues involved. For example, negatively charged amino acids include aspartic acid and glutamic acid; positively charged amino acids include lysine and arginine; amino acids with uncharged polar head groups or nonpolar head groups having similar hydrophilicity values include the following: leucine, isoleucine, valine, glycine, alanine, asparagine, glutamine, serine, threonine, phenylalanine, tyrosine. Other contemplated variations include salts and esters of the afore mentioned polypeptides, as well as precursors of the aforementioned polypeptides, for example, precursors having N-terminal substitution such as methionine, N-formylmethionine used as leader sequences. Such variations may be made without necessarily departing from the scope and the spirit of the present invention.

According to procedures known in the state of the art or according to the procedures given in the examples section, it is possible to obtain polynucleotide sequences coding for any of the s-GDH mutants as discussed above. The invention therefore comprises also isolated polynucleotide sequences encoding s-GDH mutant proteins as described above.

The present invention further includes an expression vector comprising a nucleic acid sequence according to the present invention operably linked a promoter sequence capable of directing its expression in a host cell.

The present invention further includes an expression vector comprising a nucleic acid sequence according to the present invention operably linked to a promoter sequence capable of directing its expression in a host cell. Preferred vectors are plasmids such as pACSGDH shown in Figures 3 and 4.

Expression vectors useful in the present invention typically contain an origin of replication, a promoter located upstream in the DNA sequence and are followed by the DNA sequence coding for all or part of s-GDH variants. The DNA sequence coding for all or part of the s-GDH variants is followed by transcription termination sequences and the remaining vector. The expression vectors may also include other DNA sequences known in the art, for example, stability leader sequences which provide for stability of the expression product, secretory leader sequences which provide for secretion of the expression product, sequences which allow expression of the structural gene to be modulated (e.g., by the presence or absence of nutrients or other inducers in the growth medium), marking sequences which are capable of providing phenotypic selection in transformed host cells, and the sequences which provide sites for cleavage by restriction endonucleases.

The characteristics of the actual expression vector used must be compatible with the host cell, which is to be employed. For example, when cloning in an *E.coli* cell system, the expression vector should contain promoters isolated from the genome of *E.coli* cells (e.g., , *lac*, or *trp*). Suitable origins of replication in *E.coli* various hosts include, for example, a ColE1 plasmid replication origin. Suitable promoters include, for example, *lac* and *trp*. It is also preferred that the expression vector includes a sequence coding for a selectable marker. The selectable marker is preferably an antibiotic resistance gene. As selectable markers, ampicillin resistance, or canamycin resistance may be conveniently employed. All of these materials are known in the art and are commercially available.

Suitable expression vectors containing the desired coding and control sequences may be constructed using standard recombinant DNA techniques known in the art, many of which are described in Sambrook et al. (1989).

The present invention additionally concerns host cells containing an expression vector which comprises a DNA sequence coding for all or part of the mutant s-GDH. The host cells preferably contain an expression vector that comprises all or part of one of the DNA sequences having one or more mutations shown in Table 1. Further preferred are the host

cells containing an expression vector comprising one or more regulatory DNA sequences capable of directing the replication and/or the expression of, and operatively linked to a DNA sequence coding for, all or part of mutant s-GDH. Suitable host cells include, for example, *E.coli* HB101 (ATCC 33694) available from Pomega (2800 Woods Hollow Road, Madison, WI, USA), XL1-Blue MRF available from Stratagene (11011 North Torrey Pine Road, La Jolla, CA, USA) and the like.

Expression vectors may be introduced into host cells by various methods known in the art. For example, transformation of host cells with expression vectors can be carried out by polyethylene glycol mediated protoplast transformation method (Sambrook et al. 1989). However, other methods for introducing expression vectors into host cells, for example, electroporation, biolistic injection, or protoplast fusion, can also be employed.

Once an expression vector containing an s-GDH variant has been introduced into an appropriate host cell, the host cell may be cultured under conditions permitting expression of the desired s-GDH variants. Host cells containing an expression vector which contains a DNA sequence coding for all or part of the mutant s-GDH are, e.g., identified by one or more of the following general approaches: DNA hybridization, the presence or absence of marker gene functions, assessment of the level of transcription as measured by the production of s-GDH mRNA transcripts in the host cell, and detection of the gene product immunologically. Preferably transformed host cells are identified by enzyme assay, e.g., colourimetric detection.

The present invention also teaches the generation and screening of s-GDH variants. Random mutagenesis and saturation mutagenesis is performed as known in the art. Variants are analyzed for substrate specificity for glucose, maltose as well as other sugars. The assay conditions chosen are adapted to ensure that the expected small enhancements brought about e.g., by a single amino acid substitution, can be measured. This has been accomplished by adjusting the assay conditions such that the wild type (or parent) enzyme activity is close to the lower detection limit. One mode of selection or screening of appropriate mutants is given in Example 3. Any change or improvement as compared over the wild-type enzyme this way can be clearly detected.

It should, of course, be understood that not all expression vectors and DNA regulatory sequences would function equally well to express the DNA sequences of the present

invention. Neither will all host cells function equally well with the same expression system. However, one of ordinary skill in the art may make a selection among expression vectors, DNA regulatory sequences, and host cells using the guidance provided herein without undue experimentation and without departing from the scope of the present invention.

The invention also relates to a process for producing s-GDH variants of the current invention comprising culturing a host cell of the invention under conditions suitable for production of the mutant s-GDH of the invention. For bacterial host cells, typical culture conditions are liquid medium containing the appropriate antibiotic and induction agent. Typical appropriate antibiotics include ampicillin, canamycin, chloroamphenicol, tetracyclin and the like. Typical induction agents include IPTG, glucose, lactose and the like.

It is preferred that the polypeptides of the present invention are obtained by production in host cells expressing a DNA sequence coding the mutant s-GDH. The polypeptides of the present invention may also be obtained by *in vitro* translation of the mRNA encoded by a DNA sequence coding for the mutant s-GDH. For example, the DNA sequences may be synthesized as described above and inserted into a suitable expression vector, which in turn may be used in an *in vitro* transcription/translation system.

An expression vector comprising an isolated polynucleotide as defined and described above operably linked to a promoter sequence capable of promoting its expression in a cell-free peptide synthesis system represents another preferred embodiment of the present invention.

The polypeptides produced e.g. by procedures as describe above, may then be isolated and purified using various routine protein purification techniques. For example, chromatographic procedures such as ion exchange chromatography, gel filtration chromatography and affinity chromatography may be employed.

One of the major applications of the improved s-GDH variants of this invention is for the use in test strips to monitor blood-glucose level in diabetic patients. Due to the insensitivity of PQQ-dependent glucose dehydrogenase towards oxygen, a system using the improved s-GDH variants is less prone to interference by oxygen than systems based on glucose oxidase. More important, since the s-GDH variants have improved specificity towards

glucose and significantly decreased relative enzymatic activity towards other sugars, the interference due maltose, galactose, and/or other related sugars which may be present in a sample to be analyzed is significantly reduced. Of course many kinds of samples may be investigated. Bodily fluids like serum, plasma, intestinal fluid or urine are preferred sources for such samples.

The invention also comprises a method of detecting, determining or measuring glucose in a sample using a s-GDH mutant according to the present invention. It is especially preferred that the improved method for detection of glucose in a sample is characterized in that said detection, determination or measurement of glucose is performed using a sensor or test strip device.

Also within the scope of the present invention is a device for the detection or measurement of glucose in a sample comprising a s-GDH mutant according to this invention as well as other reagents required for said measurement.

The s-GDH variants with improved substrate specificity of this invention can also be used to great advantage in biosensors (D'Costa, E. J., et al., Biosensors 2 (1986) 71-87; Laurinavicius, V., et al., Analytical Letters 32 (1999) 299-316; Laurinavicius, V., et al., Monatshefte fuer Chemie 130 (1999) 1269-1281) for online monitoring of glucose in a sample or a reactor. For this purpose, the s-GDH variants can, for example, be used to coat an oxygen-insensitive glassy electrode with an osmium complex containing a redox conductive epoxy network (Ye et al., 1993, supra) for more accurate determination of the glucose concentration.

There are also other possible applications of the s-GDH variants with the improved substrate specificity according to this invention. For example, these s-GDH variants may be used in an aldonic acid production process. Wild-type s-GDH has a high turnover in substrate oxidation producing gluconic and other aldonic acids. By using the s-GDH variants, which are more specific for glucose, the production of gluconic acid would result in much less byproducts. With other s-GDH variants of different substrate specificity, it is possible to produce different aldonic acids as required.

In the following examples, all reagents, restriction enzymes, and other materials were obtained from Roche Diagnostics Germany, unless other commercial sources are specified,

and used according to the instructions given by the suppliers. Operations and methods employed for the purification, characterization and cloning of DNA are well known in the art (Ausubel, F., et al., in "Current protocols in molecular biology" (1994), Wiley Verlag) and can be adapted as required by the skilled artisan.

The following examples further illustrate the present invention. These examples are not intended to limit the scope of the present invention, but provide further understanding of the invention.

Example 1

Cloning and expression of the wild-type *A. calcoaceticus* soluble PQQ dependent glucose dehydrogenase in *E. coli*

The s-GDH gene was isolated from *Acinetobacter calcoaceticus* strain LMD 79.41 according to standard procedures. The wild-type s-GDH gene was subcloned into a plasmid containing the mgl promoter for adjustabel expression (cf. Patent application WO 88/09373). The new construct was called pACSGDH (see Figures 3 and 4). The recombinant plasmids was introduced into a host organism selected from the *E.coli* group. These organisms were then cultivated under appropriate conditions and colonies showing s-GDH activity selected.

The plasmid pACSGDH was isolated from a 200 ml over-night culture of the clone mentioned above using the QIAGEN Plasmid Maxi Kit (Qiagen) according to the manufacturers protocol. The plasmid was resuspended in 1 ml bidest. water. The concentration of the plasmid was determined using a Beckman DU 7400 Photometer. The yield was 600 µg. Then the quality of the plasmid was determined by agarose gel electrophoresis.

Example 2:

Mutagenic PCR

To generate random mutations in the s-GDH-gene, mutagenic PCR (polymerase chain reaction) was performed. The pACSGDH plasmid and the DNA sequence encoding the mutated enzymes (PCR product from mutagenic PCR) were digested with the restriction

enzymes *Sph* I and *Eco* RI. The products were gel purified. The digested DNA sequences were ligated and an aliquot of the ligation reaction mixture was used to transform competent *E.coli* cells. The transformants were subsequently selected on LB plates containing ampicillin.

To assay, individual colonies were chosen, grown over night in LB medium containing ampicillin and subjected to screening (see Example 3) .

Mutagenic PCR reaction mixture:

40 ng pACSGDH

1 x buffer without MgCl2 (Roche Diagnostics GmbH, Cat. 1699 105)

dCTP, dTTP 1mM

dATP, dGTP 0.2 mM (Roche Diagnostics GmbH, Cat. 1969 064)

40 pmol GF23-Primer (5'-CGC GCA CGC GCA TGC CGC CGA TGT TC) (= SEQ ID NO: 4)

40 pmol GR23 (5'-GAC GGC CAG TGA ATT CTT TTC TA) (= SEQ ID NO: 5)

7 mM MgCl2

0.6 mM MnCl2

5 U Taq DNA polymerase (Roche Diagnostics GmbH, Cat. 1146 165)

Gene Amp PCR System 2400 (Perkin Elmer), 30 cycles: 95 °C 1 min, 45 °C 2 min, 72 °C 2 min

- Purification of the PCR products using the High Pure PCR Product Purification Kit from Roche Diagnostics GmbH (Cat. 1 732 676) according to the manufacturers protocol.

- Digestion of the PCR-fragments with 25 U *SphI* (Roche Diagnostics GmbH, Cat. 606 120) in 1 x buffer H (Roche Diagnostics GmbH, Cat. 1 417 991) at 37 °C over night;

addition of 25 U *EcoRI* (Roche Diagnostics GmbH, Cat. 703 737) and further digestion for 3.5 hours

- Digestion of 50 µg pACSGDH with 180 U *SphI* and 180 U *EcoRI* in 1 x buffer H for 4 hours at 37 °C.

- Gel electrophoresis of the digested pACSGDH and the digested fragments using agarose gels (0.8 %)

- Extraction of the DNA molecules using QIAquick Gel Extraction Kit (Qiagen, Cat. 28706) according to the manufacturers protocol

- Determination of the concentration of the fragments and the digested vector using a Beckman DU 7400 Photometer

- Determination of the quality of the purified products by agarose gel electrophoresis

- Ligation of 100 ng digested vector with 140 ng mPCR-fragments using 1 U T4-DNA-Ligase (Roche Diagnostics GmbH, Cat. 481 220) in a volume of 20 µl at 16 °C over night

- Electroporation of electrocompetent XL1F- cells (Stratagene) with 1 µl of the ligation reaction with 2.5 KV in 0.2 cm cuvettes using a BioRad *E. coli* Pulser (BioRad)

- After growth in 1 ml LB at 37 °C for one hour, bacteria were plated on LB-Ampicillin Agar plates (100 µg / ml Ampicillin) and grown over night at 37 °C.

- 50 % of these clones expressing mutated s-GDH were active using the following screening method.

Example 3:

Screening

The mutant colonies on agar plates described above where picked in microtiter plates (mtp) containing 200 µl LB-Ampicillin-media/well and incubated over night at 37 °C. These plates are called master plates.

From each master plate, 5 µl sample/well was transferred to a mtp containing 5 µl per/well of B (B=Bacterial Protein Extraction Reagent; Pierce No. 78248) for cell disruption and 240 µl of 0.0556 mM pyrollo-quinoline quinone(PQQ); 50 mM Hepes; 15 mM CaCl2 pH 7.0/well for activation of s-GDH were added. To complete the formation of the holoenzyme, the mtp was incubated at 25°C for 2 hours and at 10 °C over night. This plate is called working plate.

From the working plate 2 x 10 µl sample/hole were transferred to two empty mtps. After that, one was tested with glucose and the other with maltose or other selected sugar molecules as a substrate. All sugar molecules were used in equimolar concentrations.

The dE/min was calculated and the value using glucose as substrate was set to 100% activity. The value obtained with the other sugar was compared to the glucose value and calculated in percent activity ((dE/min Maltose/dE Glucose)*100). This is equivalent to the cross-reactivity of the (mutant) enzyme.

Example 4:

Sequencing of mutant s-GDH gene from mutagenic PCR

The plasmid containing the mutant s-GDH gene that leads to 50 % maltose/glucose activity was isolated (High Pure Plasmid Isolation Kit, Roche Diagnostics GmbH, No. 1754785) and sequenced using an ABI Prism Dye Terminator Sequencing Kit and ABI 3/73 and 3/77 sequencer (Amersham Pharmacia Biotech).

Following primers were used:

Sense strand: GDH F2 : 5'-TTA ACG TGC TGA ACA GCC GG-3' (= SEQ ID NO: 6)

GDH F3: 5`-GAT GCT GAT GGG CAG AAT GG-3' (= SEQ ID NO: 7)

GDH F4: 5`-ATA TGG GTA AAG TAC TAC GC -3' (= SEQ ID NO: 8)

GDH F5: 5'-ACG ATC CAA CTT GTG GAG AG-3' (= SEQ ID NO: 9)

Antisense strand: GDH R1: 5'-CGA TTA AGT TGG GTA ACG CC-3' (= SEQ ID NO: 10)

GDH R2: 5'-ATA CGG AAA ATG ACA CCA CG-3' (= SEQ ID NO: 11)

GDH R3: 5'-GGG CCT TGT TCA GAC TGC AA-3' (= SEQ ID NO: 12)

GDH R4: 5'-CAA GAC GAC CTG ACT GAT GG-3' (= SEQ ID NO: 13)

GDH R5: 5'-CAT AAC AAC GCG TGC GGC TT-3' (= SEQ ID NO: 14)

Results:

=>6 mutations on DNA sequence level

⇨ 4 mutations on amino acid level:

at position 340 (mature enzyme) change from E to G

at position 348 (mature enzyme) change from T to S

at position 369 (mature enzyme) change from N to H

at position 413 (mature enzyme) change from S to N

Example 5:

s-GDH mutants obtained by saturation mutagenesis

The QuickChange Site-Directed Mutagenesis Kit (Stratagene, Cat. 200518) was used to substitude successively wild type amino acids at defined positions of the s-GDH-protein or

of s-GDH-mutants (plasmide purification as discribed above) with other random amino acids.

The 5'- and the 3'-primer used for mutagenesis were complementary to each other and contained NNN in a central position. These nucleotides were flanked by 12 to 16 nucleotides at each end. The sequences of the nucleotides were identical to the cDNA-strand or to the complementary cDNA-strand flanking the codon for the amino acid that had to be substituted. Instead of the codon, the primer contained NNN therefore the oligonucleotides code for every codon.

For every defined position, one PCR reaction was performed.

The PCR-reactions and the *DpnI* digestions were performed according to the manual.

After that, 1 µl of each reaction was used for the electroporation of XL1F- cells. Cells were grown and the s-GDH activity of the clones was determined as described above.

To ensure statistically that all 20 amino acids variants were screened, 200 clones were tested for each position.

The following primers where used:

for position 340 Sense strand EGF 5'-TCC AAC TTG TGG ANN N AT GAC CTA CAT TT-3' (= SEQ ID NO: 15)

Antisense strand EGR 5'- AAA TGT AGG TCA TNN NTC CAC AAG TTG GA-3' (= SEQ ID NO: 16)

for position 348 Sense strand TSF 5'- CAT TTG CTG GCC ANN NGT TGC ACC GTC AT-3' (= SEQ ID NO: 17)

Antisense strand TSR 5'- ATG ACG GTG CAA CNN NTG GCC AGC AAA TG-3' (= SEQ ID NO: 18)

for position 369 Sense strand NHF 5'-TAC TGG TTG GGA ANN NAC ATT ATT GGT TC -3' (= SEQ ID NO: 19)

Antisense strand NHR 5'- GAA CCA ATA ATG TNN NTT CCC AAC CAG TA-3' (= SEQ ID NO: 20)

for position 413 Sense strand SNF 5'-TGA TGT GAT TGC ANN NCC AGA TGG GAA TG -3' (= SEQ ID NO: 21)

Antisense strand SNR 5'- CAT TCC CAT CTG GNN NTG CAA TCA CAT CA-3' (= SEQ ID NO: 22)

Results:

The amino acid changes at positions 340, 369 and 413 didn't change the substrate specificity. Only the wobble at position 348 did yield clones with a substrate specificity from 25-100 % (maltose/glucose).

Numerous rounds of mutagenic PCR and saturation mutagenesis were performed. It was found and confirmed that positions 348 and 428 are of major importance and that exchange of other amino acids may further improve the specificity for glucose of mutated s-GDH. Representative data and positions are given in table 1.

Table 1: Examples for s-GDH-variants with improved specificity for glucose

Abbreviations: n.t. = not tested

SA = specific activity (U/mg protein) with glucose as substrate

Changed amino acid to wildtype sequence	Glucose conversion	Maltose conversion	Galactose conversion	SA
wild-type	100%	105%	50%	1000
340 E to G 348 T to S 369 N to H 413 S to N	100%	50%	25%	700
22 I to L	100%	123%	14%	178

295 Q to L 422 L to I				
348 T to D	100%	80%	n.t.	n.t.
348 T to A	100%	67%	n.t.	n.t.
348 T to G	100%	22%	20%	910
428 N to P 348 T to G	100%	8%	25%	n.t.
428 N to V 348 T to G	100%	22%	22%	n.t.
127 T to M 143 D to Q 348 T to G 428 N to P	100%	1%	32%	n.t.
76 Q to A 348 T to G	100%	17%	n.t.	n.t.
76 Q to M 348 T to G	100%	18%	n.t.	n.t.
76 Q to D 348 T to G	100%	17%	n.t.	n.t.
76 Q to P 348 T to G	100%	17%	n.t.	n.t.
76 Q to S 348 T to G	100%	17%	n.t.	n.t.
76 Q to G 348 T to G	100%	20%	n.t.	n.t.
76 Q to E 348 T to G	100%	17%	n.t.	n.t.
143 D to E 348 T to G	100%	17%	n.t.	n.t.
171 Y to H 348 T to G 308 K to N	100%	19%	n.t.	n.t.
171 Y to D 348 T to G 317 F to V	100%	18%	n.t.	n.t.
127 T to S 169 L to H 348 T to G 355 Y to H	100%	11%	n.t.	n.t.
16 N to D 120 T to S	100%	22%	n.t.	n.t.

177 Q to R 277 Y to H 348 T to G				
116 I to T 255 N to T 299 K to R 348 T to G	100%	20%	n.t.	n.t.
227 H to Y 348 T to G 438 N to S	100%	18%	n.t.	n.t.
341 M to V 348 T to G	100%	20%	n.t.	n.t.
348 T to G 349 V to G 428 N to P	100%	10%	n.t.	n.t.
171 Y to G 230 P to H 348 T to G 428 N to P	100%	3%	n.t.	n.t.
230 P to H 348 T to G 428 N to P	100%	10%	n.t.	200
227 H to F 230 P to H 348 T to G 428 N to P	100%	6%	n.t.	n.t.
143 D to E 348 T to G	100%	17%	n.t.	n.t.
143 D to E 348 T to G 428 N to P	100%	1%	32%	n.t.
169 G to X 230 P to H 348 T to G 428 N to P	100%	2%	n.t.	n.t.

Example 6:

Purification of mutant s-GDH T348G

The grown cells (LB-Amp. 37 °C) were harvested and resuspended in potassium phosphate buffer pH 7.0. Cell disruption was performed by French Press passage (700-900 bar). After centrifugation the supernatant was applied to a S-Sepharose (Amersham Pharmacia Biotec) column equilibrated with 10 mM potassium phosphate buffer pH 7.0. After washing, the s-GDH was eluted using a salt gradient 0-1 M NaCl. The fractions showing s-GDH activity were pooled, dialysed against potassium phosphate buffer pH 7.0 and re-chromatographied on re-equilibrated S-sepharose column. The active fractions were pooled and subjected to a gel filtration using a Superdex® 200 column (Amersham Pharmacia Biotec). The active fractions were pooled and stored at –20 °C.

Enzyme assay and Protein determination of mutant T348G and wildtype s-GDH

Protein determination was performed using the Protein Assay Reagent no. 23225 from Pierce (calibration curve with BSA, 30 Min. 37 °C).

The GDH samples were diluted to 1 mg protein/ml with 0.0556 mM pyrollo-quinoline quinone(PQQ); 50 mM Hepes; 15 mM CaCl2 pH 7.0 and incubated at 25°C for 30 minutes for reconstitution or activation.

After activation 50 µl of sample were added to 1000 µl of a 0.2 M citrate buffer solution (pH 5.8; at 25°C) containing 0.315 mg (4-(dimethylphosphinylmethyl)-2-methyl-pyrazolo-[1.5a]-imidazol-3-yl)-(4-nitrosophenyl)-amine (see patent US 5,484,708)/ml as a mediator and 33 mM sugar).

Extinction at 620 nm is monitored during the first 5 minutes at 25 °C.

One Unit enzyme activity corresponds the conversion of 1 mMol mediator/min under the above assay conditions

Calculation: Activity = (total volume * dE/min [U/ml]) : (ε * sample volume * 1)

(ε = coefficient of extinction; in this example $\varepsilon_{620\,nm} = 30[1^* mmol^{-1} * cm^{-1}]$).

The assay was performed with glucose, maltose and galactose (Merck, Germany).

Results:

Sample	Specific activity U/mgProtein (glucose as substrate)	% maltose/glucose conversion	% galactose/glucose conversion
wilde-type	1000	105%	50%
Mutant T348G	910	22%	20%

Example 7: Determination of glucose in the presence or absence of maltose

The wild-type and mutant T348G of s-GDH were applied for glucose determination. The reference samples contained 65 mg glucose/dl. The "test"-sample contained 65 mg glucose/dl and 130 mg/dl maltose. The same amounts of GDH activity (U/ml; see enzyme assay) were used for each assay.

In a cuvette was mixed:

1 ml 0.315 mg (4-(dimethylphosphinylmethyl)-2-methyl-pyrazolo-[1.5a]-imidazol-3-yl)-(4-nitrosophenyl)-amine ml/0.2 M citrate pH 5.8

0.015 ml sample (glucose or glucose + maltose)

The assay was started adding 0.050 ml 90 U/ml s-GDH . The change of absorption at 620 nm was monitored. After 5 minutes constant values were observed and the dE/5 min calculated. The value obtained measuring the reference sample with wild-type s-GDH was set to 100%. The other values were compared to this reference value and calculated in %.

Results:

	65 mg/dl glucose	65 mg/dl glucose and 130 mg/dl maltose
wild-type s-GDH	100%	190%
mutant s-GDH T348G	100%	130%

It can be clearly seen that the „glucose-value" measured is markedly less impaired when the mutated s-GDH is used in this determination.

List of References

Anthony C., 1996. Quinoprotein-catalysed reactions. Biochem. J. 320(3):697-711

Anthony C., Ghosh M., 1997. The structure and function of PQQ-containing quinoproteins. Curr. Sci. 72(10):716-727

Anthony, C., Biochem J 320 (1996) 697-711

Anthony, C. and Ghosh, M., Current Science 72 (1997) 716-727

Anthony, C. and Ghosh, M., Prog Biophys Mol Biol 69 (1998) 1-21

Anthony, C., Biochem Soc Trans 26 (1998) 413-7

Ausubel, F., et al., in "Current protocols in molcular biology" (1994), Wiley Verlag

Cleton-Jansen, A. M., et al., Antonie Van Leeuwenhoek 56 (1989) 73-9

Cleton-Jansen, A. M., et al., J Bacteriol 170 (1988) 2121-5

Cleton-Jansen, A. M., et al., Mol Gen Genet 217 (1989) 430-6

Davidson, V. L. in "Principles and applications of quinoproteins" (1993) the whole book, New York, Marcel Dekker

D'Costa, E. J., et al., Biosensors 2 (1986) 71-87

Dokter, P., et al., FEMS Microbiology Letters 43 (1987) 195-200

Dokter, P., et al., Biochem J 239 (1986) 163-7

Dokter, P., et al., Biochem J 254 (1988) 131-8

Duine, J. A. Energy generation and the glucose dehydrogenase pathway in *Acinetobacter* in "The Biology of *Acinetobacter*" (1991) 295-312, New York, Plenum Press

Duine, J. A., Biosens. Bioelectronics 10 (1995) 17-23

Duine, J. A., et al., Arch Microbiol 131 (1982) 27-31

Duine, J. A., Eur J Biochem 200 (1991) 271-84

Goodwin, P. M. and Anthony, C., Adv Microb Physiol 40 (1998) 1-80

Hill, D. E., et al., Methods Enzymol 155 (1987) 558-68

Igarashi, S., et al., Biochem Biophys Res Commun 264 (1999) 820-4

Kaufmann, N., et al. Development and evaluation of a new system for determining glucose from fresh capillary blood and heparinised venous blood in "Glucotrend" (1997) 1-16, Mannheim, Boehringer Mannheim GmbH

Laurinavicius, V., et al., Analytical Letters 32 (1999) 299-316

Laurinavicius, V., et al., Monatshefte fuer Chemie 130 (1999) 1269-1281

Leung, D. W., et al., Technique 1 (1989) 11-15

Matsushita, K. and Adachi, O. Bacterial quinoproteins glucose dehydrogenase and alcohol dehydrogenase in "Principles and applications of Quinoproteins" (1993) 47-63, New York, Marcel Dekker

Matsushita, K., et al., Antonie Van Leeuwenhoek 56 (1989) 63-72
Matsushita, K., et al., Biochemistry 28 (1989) 6276-80
Matsushita, K., et al., Bioscience Biotechnology & Biochemistry 59 (1995) 1548-1555
Oliphant, A. R., et al., Gene 44 (1986) 177-83
Olsthoorn, A. J. and Duine, J. A., Arch Biochem Biophys 336 (1996) 42-8
Olsthoorn, A. J. and Duine, J. A., Biochemistry 37 (1998) 13854-61
Oubrie, A. and Dijkstra, B. W., Protein Sci 9 (2000) 1265-73
Oubrie, A., et al., Embo J 18 (1999) 5187-94
Oubrie, A., et al., J Mol Biol 289 (1999) 319-33
Oubrie, A., et al., Proc Natl Acad Sci U S A 96 (1999) 11787-91
Sambrook, J., et al. - in "Molecular Cloning: A Laboratory Manual" (1989) -, Cold Spring Harbour, NY, Cold Spring Harbour Laboratory Press
Wens, R., et al., Perit Dial Int 18 (1998) 603-9
Ye, L., et al., Anal. Chem. 65 (1993) 238-41

EP 0 620 283
JP 11243949
US 5,484,708
US 5,997,817
US 6,057,120
US 6,103,509
WO 92/07953
WO 99/30152
WO 88/09373

Patents Claims

1. A mutant of the soluble form of EC 1.1.99.17 also known as PQQ-dependent soluble glucose dehydrogenase (s-GDH) said mutant characterized in that relative to the corresponding wild-type enzyme and with regard to at least one other selected sugar substrate, it has an at least two-fold increased substrate specificity for glucose.
2. The mutant according to claim 1 further characterized in that said selected sugar is selected from the group consisting of maltose and galactose.
3. The mutant according to claim 1 or 2 further characterized in that said selected sugar is maltose.
4. The mutant of PQQ-dependent s-GDH according to Claim 1 further characterized in that said substrate specificity for glucose is improved at least 3-fold.
5. The mutant of PQQ-dependent s-GDH according to Claim 1 further characterized in that said substrate specificity for glucose is improved at least 5-fold.
6. A mutant of the soluble form of EC 1.1.99.17 also known as PQQ-dependent soluble glucose dehydrogenase (s-GDH) said mutant characterized in that

   a) the substrate specific reactivity towards glucose is essentially equal to that of the wild-type enzyme, and

   b) the substrate specific reactivity towards maltose is 30% or less as compared to the wild-type enzyme.

7. The mutant according to claim 6 further characterized in that said substrate specific reactivity towards maltose is 20% or less as compared to the wild-type enzyme.
8. The mutant of a PQQ-dependent s-GDH according to any of claims 1- 7 further characterized in that the wild-type s-GDH is isolated from a strain of the *Acinetobacter* species group consisting of *A. calcoaceticus* and *A. baumannii*.
9. A mutant protein of PQQ-dependent s-GDH comprising an amino acid residue substitution at the amino acid position corresponding to position 348 of the s-GDH wild-type sequence known from *A. calcoaceticus* (SEQ ID NO: 24).
10. A mutant protein of PQQ-dependent s-GDH comprising at least two amino acid residue substitutions at amino acid positions corresponding to positions of the s-GDH wild-type sequence known from *A. calcoaceticus* (SEQ ID NO: 24), said substituted amino acid positions being selected from the group consisting of positions 16, 22, 76, 116, 120 ,127, 143, 168, 169, 171, 177, 227, 230, 231, 245, 255, 277, 295, 299, 308, 317, 341, ~~348,~~ 349, 355, 422, 428 and 438, wherein the amino acid residue T348 is replaced.

11. The mutant of claim 10 further characterized in that at amino acid positions corresponding to positions of the s-GDH wild-type sequence known from *A. calcoaceticus* (SEQ ID NO: 24) the amino acid in position 348 and at least one of the following amino acid residues 16, 116, 120, 127, 169, 171, 177, 227, 255, 277, 299, 317, 355 and 438 are substituted.
12. The mutant protein of claim 10 comprising substitutions of the amino acid residues at positions 348 and 428.
13. A mutant protein of PQQ-dependent s-GDH comprising at least three amino acid residue substitutions at amino acid positions corresponding positions of the s-GDH wild-type sequence known from *A. calcoaceticus* (SEQ ID NO: 24), said substituted amino acid positions being selected from the group consisting of positions 171, 227, 230, 245, 341, 348, 349, and 428 wherein both the amino acid residues T348 and N428 are substituted.
14. The mutant according to claim 12 or 13 further characterized in that asparagine at position 428 is substituted with an amino acid residue selected from the group consisting of leucine, proline and valine.
15. The mutant according to any of claims 9 to 14 further characterized in that threonine at position 348 is substituted with an amino acid residue selected from the group consisting of alanine, glycine and serine.
16. A mutant protein of PQQ-dependent s-GDH comprising the amino acid sequence of WPXaaVAPS (SEQ ID NO: 1), wherein said Xaa residue is an amino acid residue other than threonine.
17. The mutant protein of claim 16 further characterized in that said Xaa residue is glycine.
18. A mutant protein of PQQ-dependent s-GDH comprising the amino acid sequence of TAGXaaVQK (SEQ ID NO: 2), wherein said Xaa residue is an amino acid residue other than asparagine.
19. The mutant protein of claim 18 further characterized in that said Xaa residue is proline.
20. A mutant protein of PQQ-dependent s-GDH comprising the amino acid sequence of ADGXaaNGL (SEQ ID NO: 3), wherein said Xaa residue is an amino acid residue other than glutamine.
21. The mutant of claim 20 further characterized in that said Xaa residue is selected from the group consisting of aspartic acid, glutamic acid, methionine, proline, serine, alanine or glycine.
22. An isolated polynucleotide encoding the s-GDH mutant protein according to any of claims 9 to 21.

23. An expression vector comprising an isolated polynucleotide as defined in claim 22 operably linked to a promoter sequence capable of promoting the expression of said polynucleotide in a host cell.
24. A host cell comprising the expression vector of claim 23.
25. A process for producing s-GDH variants comprising culturing the host cell of claim 24 under conditions suitable for production of the enzyme variants.
26. An expression vector comprising an isolated polynucleotide as defined in claim 22 operably linked to a promoter sequence capable of promoting its expression in a cell-free peptide synthesis system.
27. A process for producing s-GDH variants with the construct of claim 26 in a cell-free peptide synthesis system under conditions suitable for production of the said enzyme variants.
28. A method of detecting, determining or measuring glucose in a sample using a s-GDH mutant according to any of the preceeding claims, said improvement comprising contacting the sample with the mutant.
29. The method of claim 28 further characterized in that said detection, determination or measurement of glucose is performed using a sensor or test strip device.
30. A device for the detection or measurement of glucose in a sample comprising a s-GDH mutant according to any of claims 1- 29 and other reagents required for said measurement.

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Roche Diagnostics GmbH

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